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New Technique use to differentiate between Pseudo and true coagulase Activity of *Staphylococcus aureus* isolated from Different infected Sites in Patients from Baghdad Hospitals

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الخلاصة:

تم عزل 300 عينة من سلالة العنقوديات الذهبية من الأخماج الملوثة من مناطق الجسم المختلفة للمرضى من مستشفيات بغداد.

تم إجراء اختبار لنشاط انزيم التلازن الذي تنتجه البكتريا العنقودية وقد استخدم بلازما الارنب العادي المضاف لها مثبطات الانزيمات ثرومبين (والتي ليس لها تأثير على نشاط الانزيم) إذ كانت تستخدم لاختبار معلق من البكتيريا للكشف عن ما مجموعه 300 سلالة المسببة لتخثر بلازما الأرنب.

جميع العزلات البكترية سببت تخثر بلازما الارنب في حين عند إضافة االتراسول مع الهيردين أعطى نتائج موجبة لنشاط انزيم التلازن المفروز من قبل البكتريا في 200 عزلة، في حين ان استعمال بلازما الارنب الداعة للتراسول والهيرودين قد اعطت تلازن في 100 عينة فقط، حيث من المفترض ان الانزيمات المسببة للتلازن يمكن ان تحفز تفاعل التلازن للبكتريا ولتفادي هذا التفاعل المسبب لنتائج خاطئة يجب اضافة كابح لانزيم محلل البروتين.

الهدف من البحث لتوضيح فرضية فحص نماذج المجموعة من عترات المكورات العنقودية الذهبية المولدة لانزيم التلازن الدموي الحقيقي من مختلف مصادر الاخماج الشديدة.

Abstract:

A total of 300 strains of Staphylococci isolated from various pathological sites of infection from different hospitals; these specimens derived from cases of human infections were tested for staphylocoagulase activity.

Test systems employing normal citrated rabbit plasma and the same substrate supplemented with inhibitors of thrombin and proteolytic enzymes (but not influencing the staphylocoagulase activity) were used for testing suspensions of bacteria and cell-free culture supernatants. A total of 200 strains clotted normal rabbit plasma; however addition of Trasylol and heparine resulted in positive results in 100 strains, whereas plasma supplemented with Trasylol and hirudin was coagulated definitely by only 70 strains.It is postulated that proteolytic enzymes of staphylococci interfere with staphylocoagulase-induced clotting and may simulate

coagulase-positve activity of staphylococci.To avoid such false results, attest system for detection of sse shtaphylocoagulaould include proteolytic enzyme inhibitors

The purpose of this investigation was to verify this hypothesis by testing a collection of staphylococci strains from different sources of infections against rabbit plasma in atypical coagulase test versus the same supplemented with specific inhibitors of proteases and thrombin depending on the sever sites of infections.

Keyword: Staphylococcus true coagulase, production correlated to sites of infections.

Introduction:

Staphylocoagulase is a protein produced and excreted extracellular by human strains of *Staphylococcus aureus*. Therefore, it is generally accepted as a typical feature of these microorganisms, enabling their differentiation from other similar and related bacteria[1,2].

Staphylocoagulase reacts specifically with prothrombin in a stoichiometric process. Both reactants possess no enzymatic activity, but their interaction results in formation of a stable complex of specific proteolytic activity called Staphylothrombin [3,4], it converts fibrinogen, a soluble plasma protein, into insoluble fibrin in a way similar to the action of physiologically formed thrombin.Staphylothro- Mbin differs from thrombin in susceptibility to different inhibitors being resistant to such natural inhibitors of thrombin as heparin, hirudin and anti-thrombin III. Staphylothrombin, however, is inhibited by specific antibodies and diisopropylflurophosphate, an inhibitor of serine proteases[5].

Detection of staphylocoagulase activity in staphylococci is performed routinely by mixing an overnight broth culture of attested strain with rabbit plasma diluted 1:5 with saline. The plasma serves as a source of prothrombin (coagluase-reacting factor) and fibrinogen. Clotting of plasma is detected after incubation of the mixture at 37^{o}C for 1,2,4,8 and 24 hour.

This procedure is based on the assumption that coagulation of plasma is caused exclusively by staphylocoagulase. However proenzymes of plasma such as prothrombin and plasminogen can be activated in another way. It has been shown that such proteases as trypsin or papain applied in a very low concentration are able to digest prothrombin and/or plasminogen[6].

During degradation of plasma proenzymes, they are temporarily converted enzymatically to thrombin or plasmin.This type of activation of the clotting process has been demonstrated by Bacteroides melaninogenicus enzymes[7]. The purpose of this investigation was to verify this hypothesis by testing a collection of staphylococci strains against rabbit plasma in a typical coagulase test versus the

same supplemented with specific inhibitors of proteases and thrombin depending on the sources of infections to produces true coagulase enzymes in sever cases.

Materials and Methods:

Bacterial strains:

A total 300 staphylococcal strains was isolated from different pathological sites infection in different hospitals; the specimens were cultured on blood agar plate aerobically at 37^{o} C overnight, the colonies then be identified by catalase production, anaerobic fermentation of glucose and other standard methods

Clotting of plasma:

Overnight cultures of staphylococci in heart infusion broth (Difco) were added in parallel in volumes of 0.05ml to three rows of tubes, each containing 0,5 ml of one of the following:

1 - Sterile fresh-rabbit citrated plasma diluted 1:5 with sterile saline.
2 - Plasma containing (in ml) 100 Units of aprotinin (Trasylol;Bayer) and 50 units of hirudin (Reanal) and
3 - Plasma with 100 Uunits of aprotinin and 20 Units of heparin (Polfa).

All tubes were incubated at 37^{o}C in a water bath after addition of the inoculums. Clotting of plasma was recorded after 30 minutes and 1,2,4,8 and 24 hours. Results were considered positive when a trace of clot was present.

Formation of slight precipitate present at a bottom of the tube was regarded as a weak reaction. To all tubes showing negative results after 24 h of incubation, 0.05 ml of saline containing 6 Units of thrombin (Behring Co.) was added. Eventual clotting of plasma was finally recorded after 300 minutes incubation at 37^{o} C.

Antibiotic susceptibility:

Sensitivity to 12 antibiotics was investigated according standard methods by disk diffusion technique.

Fermentation of sugars and acetoin production:

Aerobic fermentation of mannitol and maltose was tested by the method of Kloos and Schleifer 1975(2)

Clumping factor:

This test was performed exactly according standard technique in elsewhere

Bacterial filtrates:

A total of 20 selected strains with positive, weak or negative results of plasma clotting in the presence of inhibitors were cultured in 500 ml portion of heart infusion broth (Difco) at 37 ^{o} C for 18 h with intensive shaking. The bacteria were sedimented by centrifugation at 10,000 rpm for 10 minutes at 4^{o}C and the supernatants were filtered through type 11605 membrane filter of pore size 0.2 nm (Sartorius Co.), they were then stored frozen -25^{o}C.

Plasma clotting by filtrates:

Portions (0.2 ml) of filtrates were added to 0.4 ml samples of the following:

1 - Sterile fresh rabbit citrated plasma diluted 1:5 with sterile saline and
2 - Plasma containing 6 mM N-ethylenediaminetetraacetic acid (Serva), 2mM N-ethylmalemide (Serva), 100 U heparin (Polfa) per ml and 100 U of trasylol (Bayer) per ml.Tubes were inspected continuously for 8 h and then after 18 h incubation at 37°C In a water bath.

Activation of prothrombin:

This was performed by incubation of 0.1 ml of filtrates with the same volume of bovine prothrombin solution (20 U) prepared by the method of Malhand Carter 1968 [8], at 37°C for 30, 90,150 and 180 minutes. After this time 0.4 ml of a 0.2% solution of bovine fibrinogen was added to each tube, and the clotting time was recorded after further incubation at 37°C.

Proteolytic activity:

Staphylococcal filtrates were cheeked for their ability to split casein by the method of Arvidson et al 1973[9].

Staphylokinase:

Detection of this activity by flooding the plates after 18 h of incubation at 37°C with a 10% solution of perchloric acid (Merck) to detect zones of proteolysis (fibrinolysis).

Identification of S.aureus types of coagulase test reaction:

Negative: No evidence of fibrin formation
1+ Positive small unorganized clot.
2+ Positive small organized clot.
3+ Positive large organized clot.
4+ Positive entire content of tuse coagulates.

PCR technique:

The *Staphylococcus* isolates was grown on Columbia agar with 5% sheep blood (Biomérieux) for 24 h at 37°C. DNA was extracted from the colonies as described by Unal et al. (10). The 50-µl PCR mixture contained 1× PCR buffer (Perkin-Elmer Applied Biosystems, Foster City, Calif.), 2 M $MgCl_2$, 16S rRNA-specific primers (0.6 µM) and *mecA*- and *nuc*-specific primers (0.4 µM each) (Amersham Pharmacia Biotech, Roosendaal, The Netherlands), deoxynucleoside triphosphates (250 µM each; Promega, Madison, Wis.), and AmpliTaq Gold DNA polymerase (2 U; Perkin-Elmer Applied Biosystems). A DNA sample of 5 µl was used as the target in the PCR. Amplification conditions consisted of 10 min at 94°C, followed by 23 cycles of 1 min at 94°C, 1 min at 51°C, and 2 min at 72°C, with a final step of 5 min at 72°C. The DNA fragments were separated by

electrophoresis on a 1.5% agarose (Invitrogen, Merelbeke, Belgium) gel stained with ethidium bromide.

DNA purification from culture samples:

Single colonies of isolates were cultured in Luria-Bertani media and incubated for 16 h at 37^0C. An aliquot (0.1 ml) of overnight culture (108 CFU) was pelleted by centrifugation (5,000 3 g for 5 min). The bacterial pellet, resuspended in 300 ml of lysis buffer (50 mM Tris HCl [pH 8.0], 100 mM EDTA, 150 mM NaCl, 1% [vol/vol] sodium dodecyl sulfate).

containing 100 mg of lysostaphin (Sigma Chemical Co., St. Louis, Mo.) and 100 mg of RNase, was incubated at 378C for 30 min. Lysis was achieved by incubation at 378C for 30 min in the presence of 200 mg of proteinase K. Samples were treated with 1 volume of phenol-chloroform-isoamyl alcohol (25:24:1) and then with 1 volume of chloroform-isoamyl alcohol (24:1) prior to precipitation of the aqueous phase in 2 volumes of 95% ethanol–0.2 M NaCl for 1 h at2208C. DNA was pelleted by centrifugation (12,500 3 g for 10 min), washed with 80% ethanol, air dried, and resuspended in 200 ml of distilled water.

PCR amplification:

Ten microliters of DNA samples was added to 90 ml of PCR mixture consisting of 10 mM Tris HCl (pH 8.8), 1.5 mM MgCl2, 50 mM KCl, 0.1% Triton X-100, 0.25 mM (each) deoxynucleoside triphosphates, 100 pmol of each primer, and 1.25 U of DyNAzyme DNA polymerase (Finnzymes, Inc., Espoo, Finland). After an initial denaturation step (3 min at 928C), 30 cycles of amplification were performed as follows: denaturation at 928C for 1 min, annealing at 568C for 1 min, and DNA extension at 728C for 1 min with an increment of 2 s per cycle. The reaction was achieved with a final extension at 728C for 3 min. Amplification was carried out in a GeneE thermal cycler (Techne, Cambridge, United Kingdom). After amplification, 15 ml of PCR samples was loaded on a 2% (wt/vol) agarose gel and horizontal electrophoresis was performed in 0.1 M Tris HCl (pH 8.6)–80 mM boric acid–1 mM EDTA containing 0.5 mg of ethidium bromide per ml. Amplified, ethidium bromide-stained DNA fragments were then visualized on a UV transilluminator at 300 nm. BglIand HinfI-cleaved pBR328 DNA fragments were used as molecular weight markers. In a first step, the specificity of the M1 and M2 primer set was assessed by using methicillin-susceptible and -resistant staphylococcal isolates from clinical sources.

Results:

All tested strains (table 1,2) clotted rabbit plasma in 18h.Addition of Trasylol (inhibitor of plasmin and other proteases) and hirudin (inhibitor of thrombin) markedly decreased the number of clotting-positive strains and increased the number of strains with weak or negative results of the clotting test. Addition of

rabbit plasma of the same amount of Trasylol and heparin inhibitory for thrombin action resulted in an increased number of weakly positive, but not negative results of the clottingtest. Thrombin added to all negative samples containing either plasma alone or plasma with inhibitors always produced a strong positive clotting reaction.

Most of the strains producing weak or negative results in the plasma clotting test in the presence of inhibitors required more time to coagulate diluted plasma without added inhibitors than did the strains with coagulating activity unaffected by inhibitors (table 3,4).

All 300 staphylococcal strains coagulating rabbit plasma also produced the clumping factor and acetoin. Most of them anaerobically fermented glucose and mannitol, and only three strains were glucose or mannitol negative. Two strains were simultaneously both glucose and mannitol negative. Two coagulase –negative strains fermented glucose and mannitol. They were mostly isolated from sever wounds and deep purulent lesions (53.0. %), but 28.1% were derived from bones and burns infection, 18.9% were from infected otitis and the remaining strains were from urinary tract infection.

A majority of strains were resistant to penicillin and Tetracycline. No correlation between plasma clotting in the presence of inhibitors and sensitivity to antibiotics, origin of strains, or glucose and/or mannitol fermentation was found. Filterates of cultures of 20 staphylococcal strains selected on the basis of their positive, weak or negative results of clotting in presence of Trasylol and hirudin were also checked for clotting abilities on rabbit plasma alone or plasma supplemented with mixture of four known inhibitors of different proteolytic enzymes (table-3). Distinct differences in clotting times between tested filtrates were noted. The 10 most active filtrates clotted plasma in 45 to 90 minutes, whereas four other strains formed a clot after 14 hours. As has been demonstrated before with whole staphylococcal cultures, only the fast-coagulating filtrates clotted rabbit plasma containing various inhibitors. Clotting time of plasma with inhibitors increased two or three times compared with results of the test with plasma alone. All tested filtrates exhibited fibrinolytic activity on fibrin plates. However, they differed in diameters of the lysis ones. Filtrates of strains with coagulating properties unaffected by inhibitors produced smaller zones than those of the strains with completely inhibited coagulase activity (table 5,6). No trace of lysis was found on the plates after heating them for 60 minutes at 80^{o}C .Various proteolytic activities were demonstrated on agarose-casein plates. Nearly all filtrates produced zones of proteolysis in three different tests. Only the filtrates strains from urine culture were completely inactive. Incubation of the filtrates with bovine prothrombine for various periods of time uniformly caused its activation, resulting in clotting of fibrinogen added thereafter. The clotting time was always

inversely related to incubation time, and after 3 h was as short as 2 to 10 minutes. No distinct differences between filtrates of various strains were found in this type of experiment (table-3).

Most of these strains could also be differentiated from *S. aureus* on the basis of their cell diameter (1 to 1.5 sum) the tube coagulase reaction of several of the strains was also tested in horse coagulase plasma EDTA and in coagulase plasma EDTA (BBL). The results are presented in Table (2). The observed difference in coagulase reaction between the various plasmas undoubtedly contributes to the difficulty and confusion in interpreting the test reaction. These results do demonstrate that the tube coagulase test is a valid means of identifying *S. aureus*. Only a complete, firm clot which does not move when the tube is tipped on its side can be considered a positive coagulase test for the purpose of identifying *S. aureus*.

The PCR results for *nuc* and *mecA*detection were in agreement with those of conventional methods for 100 and 98% of strains, respectively. The staphylococcal 16S rRNA sequence was amplified for 98% of the strains. Discrepancies between the sources from otitis media and urinary tract in the absence of an amplified *mecA*gene fragment was observed (Table-7). A weak *nuc*PCR signal was noted with *urethral source* and urinary tract. Most of the *S. aureus* from sever infected sources carry a genetic element closely related to the *mecA* gene [11]. Therefore, our PCR results suggest that sequence variations in the Staphylococci isolated from these sever cases *mecA*gene are probably localized in the region recognized by the *mecA*-specific primers. *mecA*failed to be amplified from isolates of Staphylococci isolated from otitis and urinary tract infection sites for which oxacillin MICs were 64μg/ml, indicating that this borderline resistance is mediated by a mechanism other than *mecA.* PCR assay that targets three genes: *mecA*, a determinant of methicillin resistance; *nuc*, which encodes the *S. aureus*-specific region of the thermonuclease gene; and a genus-specific 16S rRNA sequence used as an internal amplification control for staphylococcal DNA. The objectives of this study were to evaluate the accuracy of characterization of staphylococci by the 16S rRNA-*mecA*-*nuc* triplex PCR directly from blood cultures and to compare its diagnostic performance with that of conventional methods in terms of specificity, sensitivity, and detection time.

Discussion:

The present study indicates that the ability to clot plasma can be inhibited in some staphylococcal cultures by substances inactive against true staphylocoagulase or its substrate but possessing a property of blocking proteolytic enzymes, moreover, purified bovine prothrombine can be activated by staphylococcal filtrates .This may suggest that some extracellular factors of staphylococci other than staphylocoagulase and which are unable to form staphylothrombin can

exert a clotting effect. A relatively long plasma clotting time by staphylococcal strains, the staphylocoagulase activity of which is blocked by inhibitors, also indicates the presence of enzymes different from staphylocoagulase, because the dynamics of their production seem to be longer than that of coagulase (which is present in considerable quantities during the first hours of incubation.It is possible that diluting the rabbit plasma used for the stapylocoagulase test increases the clotting effect of proteases because of simultaneous dilution of natural protease inhibitors present in fresh plasma.It seems therefore, that addition of protease inhibitors to the staphylocoagulase test is a proper way to determine true staphylocoagulase activity and not the clotting produced by proteolytic enzymes of staphylococci. The protease enzymes possess narrow substrate specificities and some of them are inhibited by ethylenediaminetetraacetic acid or di-isopropy LfluoroPhos-phate.

It impossible at present to determine which protease plays arole in clotting and fibrinolysis simulating staphylocoagulase and staphylokinase activities. Because it seems very probable that the clotting is an affect of interactions of several staphylococcal enzymes, we have used whole bacterial cultures and a mixture of different protease inhibitors. The mixture of Trasylol and hirudin exhibited stronger inhibitory properties for nonspecific clotting than did the mixture of Traylol and heparin. These differences can be explained no only by application of different enzyme concentrations, but also on recent evidence that commercial preparation of hirudin contain, besides their basic enzymatic activity, some inhibitors of various proteases. It should be stressed that the presence of fibrinogen as a substrate for clotting must be verified in every negative coagulase test by addition of thrombin because in spite of coagulase activity, fibrinogen partially cleaved by protease and/or staphylokinase-induced plasmin cannot form a fibrin clot.

Application of protease inhibitors in the staphylocoagulase test excludes this danger as proteolytic enzymes are blocked by the inhibitors and staphylokinase-produced plasmin inhibited by trasylol.The question arises as to whether staphylococcal strains unable to clot rabbit plasma in the presence of inhibitors of proteolytic enzymes can be classified as *staphylococcus* aureus. The results of experiments presented in this paper suggest that not just staphylocoagulase can clot rabbit plasma under conditions generally used for detection of this factor. It is very probable that some staphylococcal proteolytic enzymes can in identical conditions; simulate both staphylocoagulase and staphylokinase by activation of prothrombin and/or plasminogen by limited proteolysis. A more precise test for detection of staphylocoagulase is needed to diminish or eliminate the possibility of obtaining false-positive or false-negative results due to other staphylococcal extracellular products present in cultures of staphylococci.

The differentiation of *S. aureus* from other Staphylococcus species of clinical interest is based mainly on the ability of *S. aureus* to clot plasma. This property can be demonstrated by the tube test for free coagulase or by the slide test for bound coagulase. The tube test is thought to be more accurate [12]. The slide coagulase test [13] performed on both types was positive, using rabbit plasma with EDTA (BBL Microbiology Systems, Cockeysville, Md.). However, the tube coagulase test [13] with rabbit plasma was positive only for the hemolytic strain (table-4). No clot of rabbit plasma was observed for the nonhemolytic strain at 1, 2, 4, 8, 24, and 48 h of incubation at 35°C (32 h) and after overnight incubation at 25°C [13]. The relative clumping factor for both types of *S. aureus* was the same, demonstrating bacterial clumping at 10 ug of fibrinogen per ml. The minimal dilution of human fibrinogen showing compact-colony formation was >1:32 for both *S. aureus* types. Further characterization of both types indicated that they were *S. aureus*. Both exhibited DNase [14] and thermonuclease [15] and were sensitive to 2,ug of lysostaphin per ml.

Acid was produced anaerobically from glucose and mannitol, and both strains grew in 10% sodium chloride. The organisms were also biochemically identical on the Staph-Ident system (Analytab Products, Plainview, N.Y.), with positive reactions for phosphatase; 3-glucosidase; urease; acidification of mannose, mannitol, and trehalose.

In the past 5 years we have noted confusion and difficulty in the interpretation of these reactions, especially the 1+ through 3+ reactions.

Further investigation into the identity of several organisms causing these reactions revealed that they were not *S. aureus* but micrococci. These observations prompted us to study the validity of the coagulase test in the identified the 4+ reaction is a very firm, opaque clot which remains in place when the tube is tipped on its side. The typical 2+ and 3+ reactions we encountered are not as opaque as the 4+ reaction and are surrounded by clear plasma.

However, no internal amplification control was included in the two assays described above. The region of 16S rRNA selected used as a positive control in the PCR assay described here was adequate for the detection of the most important species of staphylococci encountered in humans.

The PCR assay described here provided results, on average, 18 to 42 h faster than the conventional identification and susceptibility testing methods used in our laboratory, which required 24 to 48 h after detection of colonies on blood cultures, whereas the PCR required 6 h

In conclusion, the 16S rRNA-mecA-nuctriplex PCR is a good tool for rapid characterization of staphylococci in positive cultures. It confirm that true coagulase enzyme produce by Staphylococcus aureus usually present in sever infections sites of body.

Further evaluations of the clinical impact and the cost-effectiveness of this rapid diagnostic test on the management of staphylococcal bacteremia are under way.

Test System	No. of strains clotting plasma in 18 h			
Rabbit plasma diluted with normal saline	**Diluted**	**positive**	**weak**	**Negative**
	1:5	200	0	0
Rabbit plasma diluted with saline with Trasylol and hirudin	1:5	100	32	68
Rabbit plasma diluted with saline with Trasylol and Heparin	1:5	100	81	19

Table-1: Clotting of Rabbit plasma by 300 Staphylococcal strains in the presence of inhibitors of proteolytic enzymes.

Strain source of isolates	Clotting time(min)with:	
	Plasma alone	**Plasma +EDTA+NEM+heparin+Trasylol**
Wound infection	20	75
Bone infection	45	110
Burn infection	60	130
Urethral infection	90	160
Otitis infection	150	180
Urinary tract infection	150	188

Table-2: Clotting rabbit plasma by staphylococcal culture filtrates in the presence of inhibitors of proteolytic enzymes.

Strain source of isolates	Clotting time(min)after addition of bovine fibrinogine to filtrates incubated with prothrombin for				
	0 min	30 min	90 min	150 min	180 min
Wound infectio	120	98	29	21	10
Bone infection	120	80	30	6	4
Burn infection	145	100	25	13	7
Urethral infection	150	108	20	17	9
Otitis media	143	100	35	9	6
Urinary tract infection	120	90	25	3	2
Control	>360	>360	270	210	135

Table-3: Activation of bovine prothrombin by Staphylococcal culture filtrates

Site of infection	Coagulase test reaction		
	Rabbit plasma(Difco)	Rabbit plasma (BBL)	Horse plasma
Sever wound infection	+ve	+ve	+ve
Sever bone infection	+ve	+ve	+ve
Sever Burn infection	+ve	+ve	+ve
Sever Urethritis infection	+ve	+ve	+ve
Sever Otitis media	+ve or -ve	-ve	-ve
Sever UTI	-ve	-ve	-ve

Table-4: Effect of various coagulase plasma (with EDTA) on coagulase test reaction from bacteria isolated from different sites of infection

Strains from site of infection	Biochemical chracteristic				
	Coagulase reaction	Thermonucleus production(coagulase plasmaEDTA) Difco	Lysostaphin sensitivity	Anaerobic fermentation	
				Glucose	Mannitol
Wound infection	+ve	+ve	+ve	+ve	+ve
Bone infection	+ve	+ve	+ve	+ve	+ve
Burn infection	+ve	+ve	+ve	+ve	+ve
Urethral infection	+ve	+ve	Weak or –ve	-ve	-ve
Otitis media	+ve	+ve	-ve	-ve	-ve
Urinary tract infection	-ve	-ve	-ve	-ve	-ve

Table-5: Identification of strains of Staphylococci by the coagulase reaction and with biochemical characteristic.

Biochemical character	Staphylococcus aureus from different sites					
	Wound infection	Bone infection	Burn infection	Urethral infection	Otitis media	Urinary tract infection
Colony pigment	+ve	+ve	+ve	+ve	delyed	-ve
Staphylocoagulase	+ve	+ve	+ve	+ve	-ve	-ve
Clumping factor	+ve	+ve	+ve	+ve or -ve	-ve	-ve
Heat-solubile nuclease	+ve	+ve	+ve	+ve	-ve	-ve
Alkaline phosphatase	+ve	+ve	+ve	+ve	+ve	+ve
Pyrrolldonyl arylamaldase	-ve	-ve	-ve	-ve	-ve	-ve
Omlihinic decarboxylase	-ve	-ve	-ve	-ve	delyed	-ve
Urease	delyed	-ve	delyed	-ve	+ve	+ve
B-glactosidase	-ve	-ve	-ve	-ve	-ve	-ve
Acetoin production	+ve	+ve	+ve	+ve	+ve	+ve
Novobiocin resistance	-ve	-ve	-ve	-ve	-ve	-ve
Polymyxin resistance	+ve	+ve	+ve	+ve	+ve	+ve
Trehalose	+ve	+ve	+ve	-ve	-ve	-ve
Mannitol	+ve	+ve	+ve	-ve	-ve	-ve

Table -6: Biochemical characters of Staphylococcus aureus isolated from different sites of human infections.

Strain(s)	No. of isolate	PCR results[a]			Oxacillin resistance	
		16S rRNA	*mec*A	*nuc*	Diffusion test[b]	MIC or MIC range (µg/ml)
Wound infection	**20**	**+ve**	**+ve**	**+ve**	**Resistance**	**128**
Bone infection	**20**	**+ve**	**+ve**	**+ve**	**Resistance**	**128**
Burn infection	**20**	**+ve**	**+ve**	**+ve**	**Resistance**	**128**
Urethral infection	**20**	**+ve**	**+ve**	**-ve**	**Resistance**	**128**
Otitis media	**20**	**+ve**	**-ve**	**-ve**	**Resistance**	**64**
Urinary tract infection	**20**	**+ve**	**-ve**	**-ve**	**Resistance**	**64**

Table-7: Bacterial strains used for preliminary validation of 16S rRNA-*mecA*-*nuc* triplex PCR.

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